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(54) Method and compounds for solid phase synthesis of oligonucleotides and oligonucleotide analogs

Verfahren und Verbindungen für die Festphase-Synthese von Oligonukleotiden und Analogen

Méthode et composés pour la synthèse en phase solide d'oligonucléotides et ses analogues

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(73) Proprietor: **POLISH ACADEMY OF SCIENCES, Center of Molecular and Macromolecular Studies**
Sienkiewicza 112 (PL)

(72) Inventors:
- **Stec, Wojciech, Jacek**
  P-95-054 Ksawerow (PL)
- **Grajkowski, Andrzej**
  P-93-533 Lodz (PL)
- **Uznanski, Bogdan**
  P-91-511 Lodz (PL)

(74) Representative: **West, Alan Harry et al**
R.G.C. Jenkins & Co.
26 Caxton Street
London SW1H 0RJ (GB)

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Description

The invention relates generally to the solid phase synthesis of oligonucleotides, and more particularly, to methods and compounds for synthesizing P-chiral oligonucleotide analogs.

The development of reliable and convenient methods for solid phase synthesis of polynucleotides has led to many advances in molecular biology and related fields, e.g. Itakura, Science, Vol. 209, pgs. 1401-1405 (1980); Caruthers, Science, Vol. 230, pgs 281-285 (1985); and Eckstein, ed., Oligonucleotides and Analogues: A Practical Approach (IRL Press, Oxford, 1991). In particular, the availability of synthetic oligonucleotides and a variety of nuclease-resistant analogs, e.g. phosphorothioates, methylphosphonates, and the like, has encouraged investigation into their use as therapeutic compounds for treating a variety of conditions associated with the inappropriate expression of indigenous and/or exogenous genes, e.g. Cohen, Ed., Oligonucleotides: Antisense Inhibitors of Gene Expression (Macmillan Press, New York, 1989); Van der Krol et al, Biotechniques, Vol. 6, 958-976 (1988); Matsukura et al, Proc. Natl. Acad. Sci., Vol. 86, pgs. 4244-4248 (1989); Iyer et al, Nucleic Acids Research, Vol. 18, pgs. 2855-2859 (1990); Leiter et al, Proc. Natl. Acad. Sci., Vol. 87, pgs. 3430-3434 (1990); McManaway et al, Lancet, Vol. 335, pgs. 808-811 (1990); Manson et al, Lymphokine Research, Vol. 9, pgs. 35-42 (1990); Sankar et al, Eur. J. Biochem., Vol. 184, pgs. 39-45 (1989); Agrawal et al, Proc. Natl. Acad. Sci., Vol. 86, pgs. 7790-7794 (1989); Miller, Biotechnology, Vol. 9, pgs. 358-362 (1991); Chiang et al, J. Biol. Chem., Vol. 266, pgs. 18162-18171 (1991); Calabretta, Cancer Research, Vol. 51, pgs. 4505-4510 (1991), and the like. Usually, these compounds are employed as "antisense" compounds. That is, the compounds are oligonucleotides, or analogs thereof, that have a base sequences complementary to segments of target nucleic acids, usually RNAs, such that duplexes or triplexes form that either render the respective targets more susceptible to enzymatic degradation, block translation or processing, or otherwise block or inhibit expression, e.g. Cohen (cited above); Moser et al, Science, Vol. 238, pgs. 645-650 (1987).

Many of the phosphate-analog linkages of these antisense compounds, as well as those of related non-nucleosidic polymers, are chiral at the phosphorus, e.g. phosphorothioates, phosphoroselenoates, methylphosphonates, and the like, Zon, Pharmaceutical Research, Vol. 5, pgs. 539-549 (1988); and Uhlmann and Peyman, Chemical Reviews, Vol. 90, pgs. 543-584 (1990). Currently, there is no way to control the chirality of these phosphorus moieties during solid phase synthesis. Consequently, the synthesis of such polymers results in mixtures of diastereoisomers, wherein the individual polymers of the mixtures have random sequences of R_p and S_p chiral phosphorus linkages along their backbones. Such mixtures prepared by currently available technology consist of 2^n diastereoisomers, where n is the number of P-chiral linkages in the polymer. For example, a trimer with two P-chiral linkages has 2^2=4 possible diastereoisomers, indicated by the following 5'->3' sequences of linkages: R_p-R_p, R_p-S_p, S_p-R_p, and S_p-S_p. In addition to the lack of methods for synthesizing polymers of predetermined chirality, there is also a lack of available analytical tools for direct measurement of the reproducibility of preparing a diastereoisomer population of polymers having P-chiral linkages for anything greater than 4-mers, Zon, pgs. 301-349 in Hancock, Ed., High-Performance Liquid Chromatography in Biotechnology (John Wiley, New York, 1990). The inability to prepare oligonucleotide analogs and related non-nucleosidic polymers with predetermined sequence, length, and chirality is problematic because there is strong evidence that chirality is an important factor in duplex stability and nuclease resistance, e.g. Lesnikowski et al, Nucleic Acids Research, Vol. 18, pgs. 2109-2115 (1990); Burgers et al, J. Biol. Chem., Vol. 254, pgs. 7476-7478 (1979); Miller et al, Biochem., Vol. 18, pgs. 5134-5143 (1979); Zon, Pharmaceutical Research (cited above); Eckstein, Ann. Rev. Biochem., Vol. 54, pgs. 367-402 (1985); and the like. This evidence suggests that stereo-controlled synthesis of antisense and related compounds with predetermined chirality at each P-stereogenic center might allow one to design particular therapeutic diastereoiscmers which fcrm maximally stable duplexes and which are maximally resistant towards nucleolytic enzymes. thus increasing their effective life times, and in this way decreasing the required amount of xenobictic material fcr a given therapeutic effect.

The invention is directed to a new method of synthesizing oligonucleotides and related polymers whose monomeric units are linked by phosphate groups, or analogs thereof. In a preferred embodiment of the invention, the method includes synthesis of such compounds with predetermined chirality whenever the phosphorus linkages are chiral. The invention includes synthons and synthon precursors for making such polymers, as well as the polymers themselves whenever the phosphorus linkages are chiral and their sequence of chirality predetermined. The synthons of the invention are hydroxyl-protected monomer-O-[1,3,2-dichalcogen-substituted-phospholane]s which may be separated into their R_p and S_p chiral forms, where appropriate, to permit solid phase synthesis of a polymer having a predetermined sequence of R_p or S_p linkages.

The method of the invention comprises the following steps: (a) providing a first monomer attached to a solid phase support, the first monomer having a protected hydroxyl; (b) deprotecting the protected hydroxyl to form a free hydroxyl; (c) reacting with the free hydroxyl in the presence of a catalytic base a synthon selected from Formula I (below); and (d) repeating steps (b) and (c) until a polymer of predetermined length is obtained. Preferably, the method further comprises the step of capping unreacted hydroxyl groups after the step of reacting. An important feature of the method of the invention is to attach the first monomer to a solid phase support by a linking group which is not cleaved in the

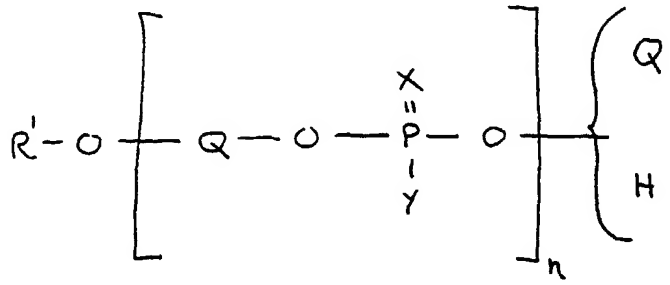
presence of the catalytic base.

Preferably, whenever a P-chiral linkage is formed between monomers, step (d) comprises repeating steps (b) and (c) until a polymer of predetermined length and chirality is obtained. Most preferably, whenever a P-chiral linkage is formed between monomers and the monomers are nucleosides or analogs thereof, step (c) includes selecting the desired P-chiral form of the synthon of Formula I and step (d) comprises repeating steps (b) and (c) until a P-chiral oligonucleotide of predetermined length is obtained.

The present invention provides a new chemistry for synthesizing oligonucleotides and related polymers having phosphate, or phosphate analog, linkages. In particular, whenever the phosphate analog linkages are P-chiral, the present invention provides a method of synthesizing polymers having a predetermined sequence of P-chirality along the polymer backbone. P-chiral oligonucleotides of the invention can be employed as antiviral agents, antisense therapeutic compounds, hybridization probes, and the like.

The invention provides a novel approach to solid phase synthesis of oligonucleotides and related polymers using hydroxyl-protected monomer-O-[1,3,2-dichalcogen-substituted-phospholane] synthons. In particular, the invention includes 2-N-substituted-1,3,2-dichalcogen-substituted-phospholane precursors of the above synthons, the hydroxyl-protected monomer-O-[1,3,2-dichalcogen-substituted-phospholane] synthons themselves, and P-chiral oligonucleotides and related P-chiral polymers having lengths in the range of 4 to several hundred monomers, and preferably, in the range of 12 to 60 monomers.

Polymers synthesized by the method of the invention generally have the formula:

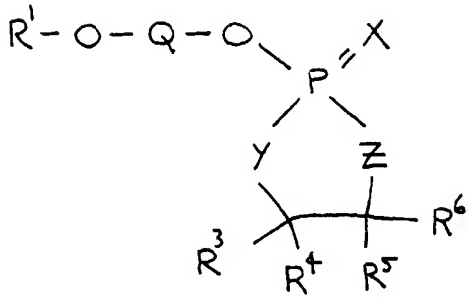


Formula I

wherein: Q is an alkyl or alkenyl having from 1 to 8 carbon atoms; an alkyloxy or alkylthio having from 1 to 8 carbon atoms and from 1 to 2 oxygen or sulfur heteroatoms; or when taken together with both adjacent oxygens, is a nucleoside or a nucleoside analog. Preferably, Q is alkyl or alkenyl having from 3 to 6 carbon atoms, or alkoxy having from 3 to 6 carbon atoms and one oxygen atom, or when taken together with both adjacent oxygens, is a nucleoside or a nucleoside analog. More preferably, Q is alkyl having from 3 to 6 carbon atoms, or a cyclic alkoxy having from 4 to 5 carbon atoms and one oxygen atom, or when taken together with both adjacent oxygens, is a nucleoside or a nucleoside analog. R^1 is hydrogen or a hydroxyl protecting group, such as triphenylmethyl (i.e., trityl), p-anisyldiphenylmethyl (i.e., monomethoxytrityl or MMT), di-p-anisylphenylmethyl (i.e., dimethoxytrityl or DMT), pivaloyl, acetyl, 4-methoxytetrahydropyran-4-yl, tetrahydropyranyl, phenoxyacetyl, isobutyloxycarbonyl, pixyl, benzyl, trialkylsilyl having from 3 to 9 carbon atoms, 9-fluorenylmethyl carbamate (Fmoc), or the like. Greene and Wuts, Protective Groups in Organic Synthesis, 2nd Edition (John Wiley, New York, 1991) provides extensive guidance on the selection of protecting groups for the various embodiments of the invention.

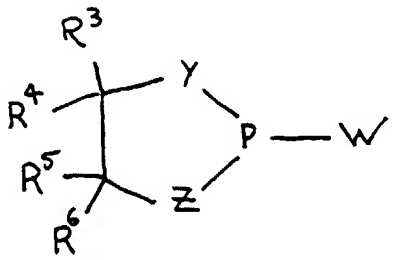
X is chalcogen, preferably S, O, or Se, or a substituted imino of the form $=NR^2$ wherein R^2 is alkyl having from 1 to 6 carbon atoms or R^2 is aryl, alkyl-substituted aryl, or alkenyl-substituted aryl having from 6 to 12 carbon atoms. Y is chalcogen, preferably S, O, or Se. n is in the range of 1 to several hundred. Preferably, n is in the range of 5-200; more preferably, n is in the range 12-60; and most preferably, n is in the range of 15-30.

The synthons of the invention are hydroxyl-protected monomer-O-[1,3,2-dichalcogen-substituted-phospholane]s, preferably defined by the formula:



Formula II

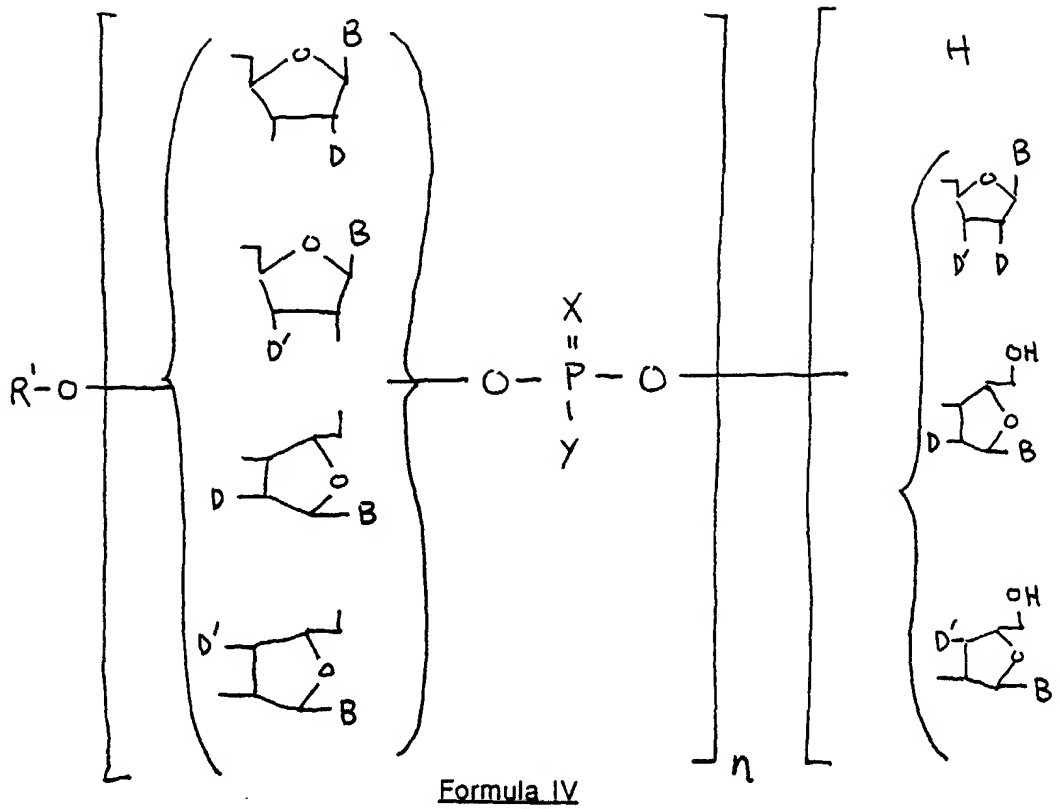
Q, R^1, X, and Y are defined as above. Z is S or Se; and more preferably, Z is S. R^3, R^4, R^5, and R^6 are separately hydrogen or an electron-withdrawing group. Preferably, R^3, R^4, R^5, and R^6 are separately alkyl having from 1 to 4 carbon atoms, or are separately or together with the carbon atoms to which they are attached aryl or alkyl-subsituted aryl having from 6 to 12 carbon atoms. Whenever a particular selection of R^3, R^4, R^5, and R^6 result in chirality, it is understood that the synthon of Formula II is used with a given stereochemical array of these ring substituents. The synthon of formula II is made by reacting the following cyclic phosphite with a free hydroxyl of an appropriate monomer:



Formula III

wherein Y, Z, and R^3 through R^6 are defined as above, and W is a leaving group amenable to nucleophilic attack by a free hydroxyl of a monomer. Preferably, W is halogen, dialkylamino having from 2 to 6 carbon atoms, morpholinyl, piperidinyl, or pyrrolidinyl. More preferably, W is selected from the group consisting of Cl, morpholino, and diisopropylamino.

P-chiral polymers of the invention are generally defined by Formula I, with the provisos that X and Y are not the same in at least one linkage and that not all linkages in the same polymer need be identical. More particularly, P-chiral oligonucleotides of the invention are defined by the formula:



Formula IV

wherein X, Y, and R^1 are defined as above, again with the proviso that X and Y are not the same in at least one linkage and that not all linkages in the same oligonucleotide need be identical. B is a natural or synthetically modified purine or pyrimidine base. D' is a 3'-hydroxyl protecting group. D is hydrogen, halogen, hydroxyl, or - OR', wherein R' is alkyl having from 1 to 3 carbon atoms or a 2'-hydroxyl protecting group, such as alkylsilyl, e.g. t-butyldimethylsilyl, or the like. Preferably, n is in the range of 5-200, and more preferably in the range of 12-60. It is understood from Formula IV that P-chiral oligonucleotides of the invention may include 5'-3', 5'-2', 5'-5', 3'-3', 2'-2', and 3'-2' linkages between nucleosides by the appropiate selection of synthons of Formula II.

The term "oligonucleotide" as used herein includes linear oligomers of natural or modified nucleosides, including deoxyribonucleosides, ribonucleosides, α-anomeric forms thereof, and the like, usually linked by phosphodiester bonds or analogs thereof ranging in size from a few monomeric units, e.g. 3-4, to several hundreds of monomeric units. Preferably, oligonucleotides of the invention are oligomers of the natural nucleosides having a lengths in the range of 12 to 60 monomeric units, and more preferably, having lengths in the range of 15 to 30 monomeric units. Whenever an oligonucleotide is represented by a sequence of letters, such as "ATGCCTG," it will be understood that the nucleotides are in 5'->3' order from left to right.

Phosphorus linkages between nucleosidic monomers include phosphodiester bonds and analogs of phosphodiester bonds, such as phosphorothioate, phosphorodithioate, phosphoroselenoate, phosphorodiselenoate, phosphoroanilidothioate, phosphoranilidate, and the like. Preferably, the monomers of the oligonucleotides of the invention are linked by phosphodiester, phosphorothioate, or phosphorodithioate linkages.

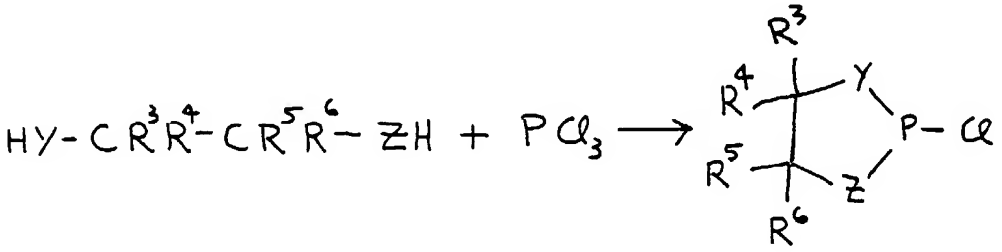
As used herein, "nucleoside" includes the natural nucleosides, including 2'-deoxy and 2'-hydroxyl forms, e.g. as described in Kornberg and Baker, DNA Replication, 2nd Ed. (Freeman, San Francisco, 1992). "Analogs" in reference to nucleosides includes synthetic nucleosides having modified base moieties and/or modified sugar moieties, e.g. described generally by Scheit, Nucleotide Analogs (John Wiley, New York, 1980). Such analogs include the natural and synthetic nucleosides with or without appropriate protecting groups for synthesis in accordance with the invention. An exemplary list of nucleoside analogs includes 2-aminopurine, deoxyinosine, N^4-methoxydeoxycytidine, N^4-aminodeoxycytidine, 5-fluorodeoxyuridine, and the like.

The term " electron-withdrawing" denotes the tendency of a substituent to attract valence electrons of the molecule of which it is apart, i.e. it is electronegative, March, Advanced Organic Chemistry, pgs. 16-18 (John Wiley, New York, 1985).

Some aspects of the invention are common to other approaches to solid phase synthesis of oligonucleotides, e.

g. selection of protecting groups. selection of solid phase supports, and the like. Consequently, considerable guidance in making such selections in the context of the present invention can be found in literature, e.g. Gait. editor, Oligonucleotide Synthesis: A Practical Approach (IRL Press, Oxford, 1984); Amarnath and Broom, Chemical Reviews, Vol. 77, pgs. 183-217 (1977); Pon et al, Biotechniques, Vol. 6, pgs. 768-775 (1988); Ohtsuka et al, Nucleic Acids Research, Vol. 10, pgs. 6553-6570 (1982); Eckstein, editor (cited above). Greene and Wuts (cited above), Narang, editor, Synthesis and Applications of DNA and RNA (Academic Press, New York, 1987), and the like.

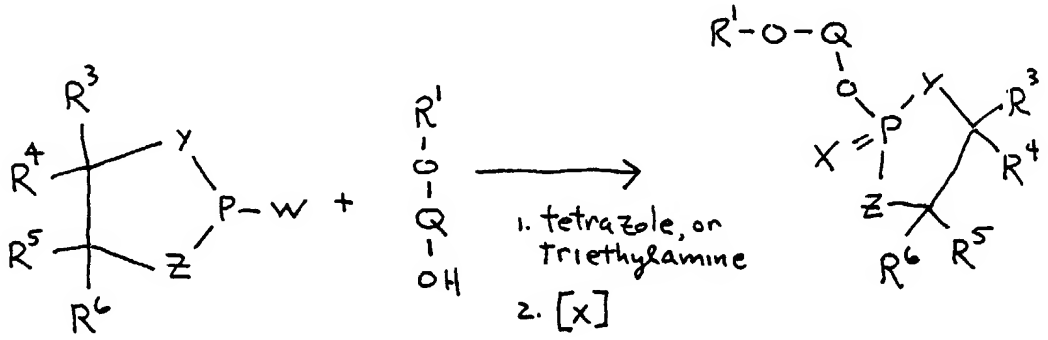
Synthon precursors of the invention are generally synthesized in accordance with the following scheme:



Scheme I

Generally, the synthon precursors are synthesized by reacting an appropriately 1,2-substituted ethane derivative with phosphorus trichloride in an aprotic solvent, preferably hexane, diethyl ether, or methylene chloride, at a temperature in the range of -10°-30°C, in the presence of a base, such as, trialkylamine or pyridine. Preferably, the base employed is pyridine. The resulting 2-chloro-1,3,2-dichalcophospholane is then reacted with secondary amines, such as N,N-diisopropylamine or morpholine, to give the preferred synthon precursors.

Synthons of the invention are generally synthesized in accordance with the following scheme:

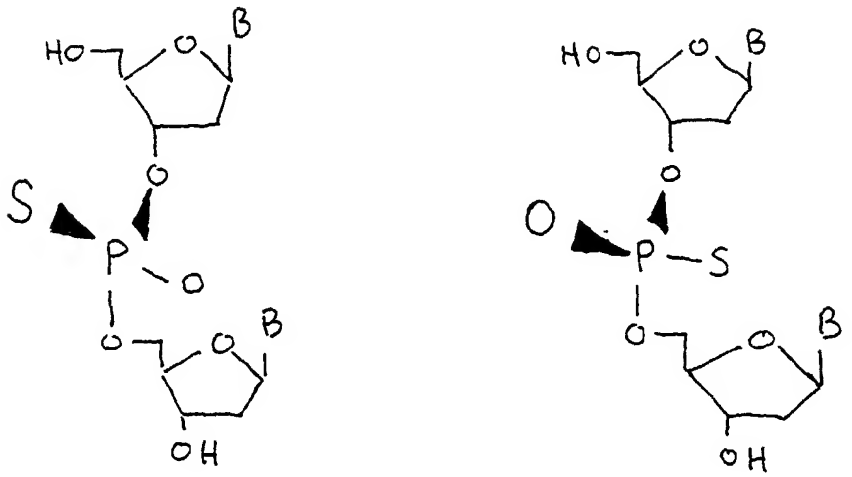


Scheme II

wherein R^1, R^3 through R^6, W, X, Q, Y, and Z are defined as above. [X] represents an agent for transferring the X moiety to the phosphorus. When X is S, [X] is elemental sulfur, 1,1 -dioxo-3H-1,2-benzodithiol-3-one, or an acyl disulfide or corresponding diphosphorothioyl disulfide, e.g. Stec et al, PCT/US91/01010. When X is Se, [X] is elemental selenium or a saturated solution of potassium selenocyanate, e.g. in 95% pyridine/5% triethylamine as taught by Stec et al, J. Amer. Chem. Soc., Vol. 106, pgs. 6077-6079 (1984). When X is NR^2, [X] is azide of the form N_3R^2, wherein R^2 is aryl having from 6 to 12 carbon atoms. Preferably, R^2 is phenyl. Preferably, the reaction is carried out in an aprotic organic solvent, e.g. methylene chloride, or like solvent, and when W is nonhalogen, is catalyzed with a mild acid, such as tetrazole or substituted tetrazole, e.g. Dahl et al, Nucleic Acids Research, Vol. 15, pgs. 1729-1743 (1987). When W is halogen, the reaction is preferably catalyzed by a mild base, such as pyridine, substituted pyridine, or trialkylamine. Preferably, the mild base is triethylamine or diisopropylethylamine.

Whenever Q, X, Y, and Z are selected so that the synthon is P-chiral, the R_p and S_p forms of the synthon must be separated prior to synthesizing polymers of predetermined chirality at the P-stereogenic centers. As used herein, "R_p" and "S_p" refer to the alternative stereo-configurations of the chiral phosphorus atoms in either the synthons or the

phosphorus linkages in the polymers. Exemplary R_p and S_p dimers are shown below:



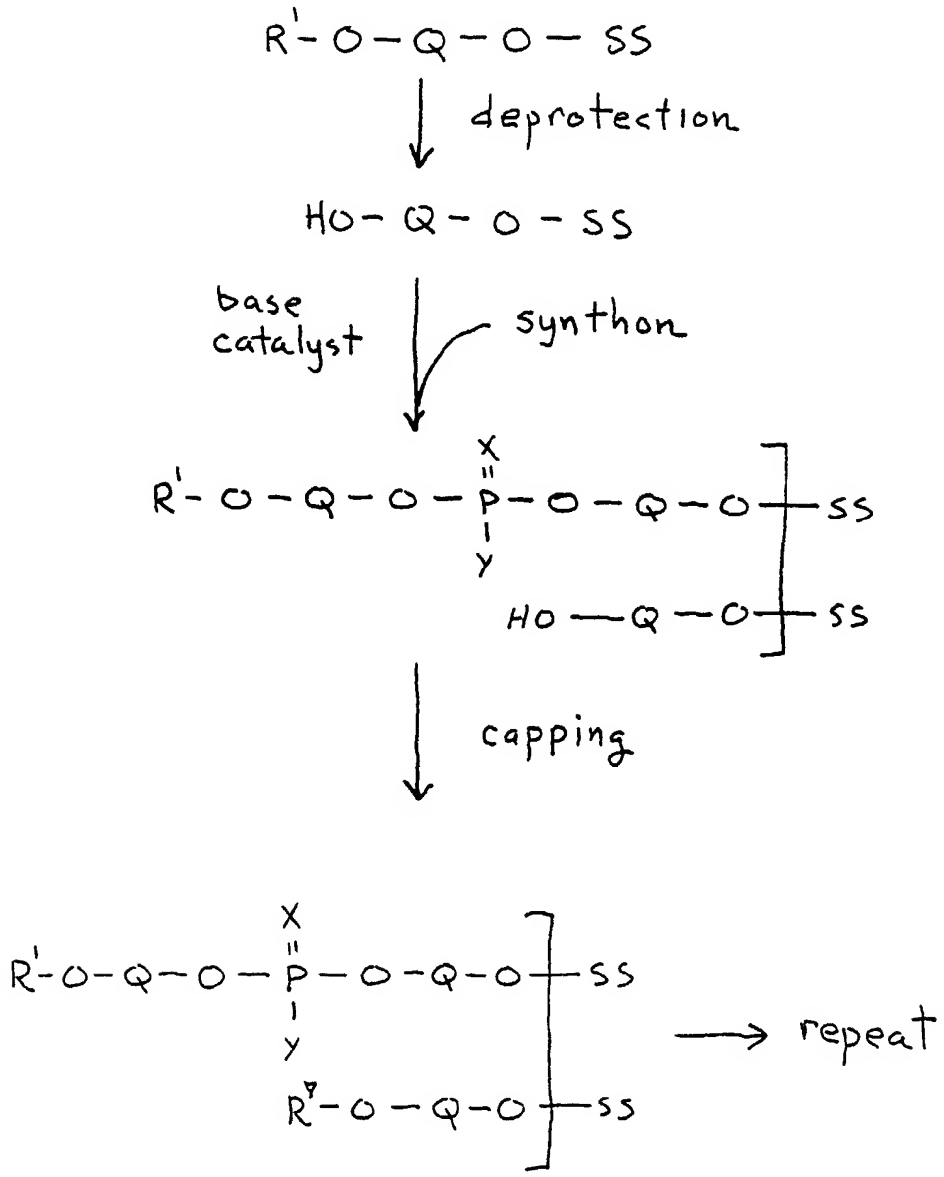
Sp Rp

In reference to P-chiral polymers, the sequence of chiral phosphorus atoms is denoted by subscripts "ps" or "pr" between monomers, e.g. $Q_{ps}Q_{ps}Q_{ps}Q_{ps}Q$ for a pentamer having four Sp phosphorus linkages, or $A_{ps}T_{pr}G_{ps}A_{pr}c_{ps}T_{pr}T_{ps}$-$G_{pr}G_{ps}A_{pr}C$ for an 11-mer oligonucleotide having alternating R_s and R_p phosphorus linkages (where A, C, G, and T represent the natural 2'-deoxynucleosides deoxyadenosine, deoxycytidine, deoxyguanosine, and deoxythymidine, respectively, unless otherwise specified). When the phosphorus linkage is achiral, no designation will be made between monomers. Thus, $Q_{ps}QQQQ$ represents a pentamer with a single P-chiral linkage of the S_p type, while the rest of the linkages are either achiral or of mixed R_p and S_p chirality.

In regard to nucleoside monomers, the phospholane moiety of the synthons can be attached to either the 3' or 5' hydroxyl, permitting either 3'->5' or 5'->3' synthesis of oligonucleotides. Preferably, the phospholane moiety is attached via the 3' hydroxyl.

Separation of the R_p and S_p chiral forms of the synthons is carried out using standard techniques, usually silica gel chromatography or high performance liquid chromatography (HPLC), e.g. Mislow, Introduction to Stereochemistry (W.A. Benjamin, New York, 1966). In regard to nucleoside synthons, in some cases the R_p and S_p forms can be identified by differential susceptibility to digestion of their oligomers to well known nucleases, but usually the R_p form is the slower eluting diastereoisomer under HPLC conditions described below. Conventional X-ray crystallography and 2-D NMR methods can also be used to assign the absolute stereochemistry at phosphorus in cases where the chirality of the monomer is known. As used herein, "diastereoisomerically pure" in reference to the R_p and S_p forms of the synthons of the invention or in reference to oligomers with a particular sequence of R_p and S_p chirality means the indicated stereochemistry essentially free of all other phosphorus configurations. Preferably, it means a compound consisting of greater than 95% of the indicated stereochemistry for each linkage, on a molar basis, and less than 5% of other phosphorus configurations for each linkage. More preferably, it means a compound consisting of greater than 99% of the indicated stereochemistry for each linkage, on a molar basis, and less than 1% of other phosphorus configurations for each linkage.

Polymers of the inventions are generally synthesized in accordance with the following scheme:



Scheme III

wherein Q, X, Y, and Z are defined as above, and wherein SS is a solid phase support and R^7 is a capping agent, e. g. acyl, isopropylphosphonate, or the like. An important feature of the coupling reaction is the presence of a catalytic base, preferably non-nucleophilic, such as potassium tert-butanolate, 1-methylimidazole, N-methylimidazole, 4-dimethylaminopyridine (DMAP), 1,8-diazabicyclo[5.4.0]undec-7-ene (D8U), or the like. Preferably, DBU is employed as the catalytic base, and is employed in large (e.g. about 100-300x) molar excess of the reactants. The coupling reaction can be performed in an anhydrous organic solvent, such as acetonitrile, methylene chloride, N,N-dimethylformamide, or the like. It is understood that SS may include a solid phase support with oligonucleotides attached that have been synthesized using a different chemistry, e.g. employing phosphoramidite synthons.

A variety of solid phase supports can be used with the present invention, but controlled pore glass or polystyrene are preferred. Preferably, the solid phase support is derivatized with the first monomer of the polymer chain to be synthesized, such that the linking group connecting the monomer to the support is stable in the presence of the catalytic

base and other reagents employed in the synthesis cycle. Preferably, polymers of the invention are attached to solid phase supports, e.g. controlled pore glass (CPG), by a sarcosinyl linker, as taught by Brown et al, J. Chem. Soc. Chem. Commun., 1989, pgs. 891-893; and Pfleiderer et al, Tetrahedron Letters, Vol. 31, pg. 2549 (1990). Briefly, the solid phase support is functionalized as follows: 9-fluorenylmethoxycarbonyl-sarcosine (FMOC-sarcosine)(10 equiv.) and dicyclohexylcarbodiimide (DCC)(15 equiv.) are added to long chain alkylamino-CPG in a mixture of N,N-dimethylformamide (DMF) and dichloromethane. Removal of the FMOC group with piperidine in pyridine is followed by coupling of the sarcosine methylamino group to a hydroxyl-protected-monomer-O-succinate (10 equiv.) in the presence of DCC (15 equiv.). For 5'-O-DMT-protected nucleosides, this results in a loading of about 20 microquiv. per gram of dry support.

Preferably, in addition to the general steps of the synthesis cycle given above, after each coupling step, a capping step is added wherein unreacted free hydroxyls are reacted with a group that prevents further monomer addition. Preferably, the unreacted free hydroxyls are reacted with capping solution consisting of one part acetic anhydride/lutidine in tetrahydrofuran (THF) (10:10:80 v/v/v) and one part N-methylimidazole in THF (16:84 v/v). More preferably, the solid phase support is washed with a suitable solvent, usually acetonitrile, after each deprotection, coupling, and capping step.

Preferably, the method of the invention is automated. The apparatus for automating can take several forms. Generally, the apparatus comprises a series of reagent reservoirs, a synthesis chamber containing the solid phase support and a computer controlled means for transferring in a predetermined manner reagents from the reagent reservoirs to and from the synthesis chamber and the purification chamber, and from the synthesis chamber to the purification chamber. The computer controlled means for transferring reagents can be implemented by a general purpose laboratory robot, such as that disclosed by Wilson et al, Bio Techniques, Vol. 6, pg. 779 (1988), or by a dedicated system of tubing, and electronically controlled valves. Preferably, the computer controlled means is implemented by a dedicated system of valves and tubing connecting the various reservoirs and chambers. In further preference, the reagents are driven through the tubing by maintaining a positive pressure in the reagent reservoirs by means of a pressurized inert gas, such as argon, as is used by many widely available automated synthesizers, e.g. Applied Biosystems, Inc. models 380B or 381A DNA synthesizers.

Oligonucleotides of the invention can be employed as hybridization probes, as taught in Hames et al, editors, Nucleic Acid Hybridization: A Practical Approach (IRL Press, Oxford, 1985).

Polymers of the invention can also be employed as components of pharmaceutical compositions. In the case of poly(alkyl or alkenyl phosphate)s of the invention, such compositions contain an antiviral therapeutic amount of at least one of the poly(alkyl or alkenyl phosphate) and/or at least one of their thiophosphate analogs in a pharmaceutically effective carrier. The poly(alkyl or alkenyl phosphate)s and their thio analogs may be administered either as a single chain length (i.e. one value of n), or as a defined mixture containing polymers of more than one chain length. Most preferably, a single chain length is employed in the range of 15 to 30 monomers.

A variety of diseases and disorders can be treated by administration of a composition comprising antisense oligonucleotides of the invention. Viral diseases that can be treated by antisense inhibition of nucleic acid expression include, but are not limited to, those caused by hepatitis B virus, cytomegalovirus, herpes simplex virus I or II, human immunodeficiency virus type I or II, influenza virus, respiratory syncytial virus, and human papilloma virus. Malignancies which can be treated by administration of antisense compounds of the invention include, but are not limited to, lung cancer (e.g., small cell lung carcinoma), colorectal cancer, prostate cancer, breast cancer, and leukemias and lymphomas. In such diseases, the antisense compounds are targeted to aberrantly expressed oncogenes associated with the diseases, or to other genes being inappropriately expressed as part of the disease condition, e.g. Aaronson, Science, Vol. 254, pgs. 1146-1153 (1991). Acute inflammatory and immune reactions, such as septic shock, eosinophilia, and the like, can also be treated with antisense compounds of the invention, wherein inappropriately and/or aberrantly expressed cytokine genes are inhibited, e.g. Tracey et al, Nature, Vol. 330, pgs. 662-664 (1987), U.S. patent 5,055,447, arid Waage et al, J. Exp. Med., Vol. 169, pgs. 333-338 (1989)(antisense TNF-α and/or TNF-β); Starnes et al, J. Immunol., Vol. 145, pgs. 4185-4191 (1990), and Fong et al, J. Immunol., Vol. 142, pgs. 2321 -2324 (antisense IL-6); Coffman et al, Science, Vol.245 pgs. 308-310 (antisense IL-5); Finkelman et al, J. Immunol., Vol. 141, pgs. 2335-2341 (1988) (antisense IL-4); Young et al, Blood, Vol. 68, pgs. 1178-1181 (1986)(antisense GM-CSF); and the like.

A pharmaceutical carrier can be any compatible, non-toxic substance suitable for delivering the compositions of the invention to a patient. Sterile water, alcohol, fats, waxes, neutral lipids, cationic lipids, and inert solids may be included in a carrier. Pharmaceutically acceptable adjuvants, e.g. buffering agents, dispersing agents, and the like, may also be incorporated into the pharmaceutical composition. For topical administration, the compositions of the invention are formulated into ointments, salves, gels, or creams, as generally known in the art. Generally, compositions useful for parenteral administration of drugs are well known, e.g. Remington's Pharmaceutical Science, 15th ED. (Mack Publishing Company, Easton, PA, 1980). Compositions of the invention may also be administered by way of an implantable or injectable drug delivery system, e.g. Urquhart et al, Ann. Rev. Pharmacol. Toxicol., Vol. 24, pgs. 199-236 (1984); Lewis, ed. Controlled Release of Pesticides and Pharmaceuticals (Plenum Press, New York, 1981); U.S. patent 3,773,919; U.S. patent 3,270,960; or the like. Compounds of the invention may also be conjugated to transport moieties

to aid in targeting tissues or in penetrating cell membranes and the like, e.g. as taught by Latham et al, PCT application WO 91/14696.

Preferably, compositions of the invention are administered parenterally, and more preferably, intravenously. In such cases, pharmaceutical carriers include saline solutions, dextrose solutions, combinations of the two, nonaqueous solutions such as ethyl oleate, and the like.

Selecting an administration regimen for a composition of the invention depends on several factors, including the rate of degradation of the particular compounds in serum, the accessibility of the target tissues and cells, pharmacokinetics, toxicity, and the like. Preferably, an administration regimen maximizes the amount of compound delivered to a patient consistent with an acceptable level of side effects. Accordingly, the amount of compound delivered may depend on the particular compound and the severity of the viral infection or other condition being treated. Preferably, a daily dose of the compounds of the invention is in the range of about 1-2 ug/kg to about 10-20 mg/kg.

Example 1

2-chloro- 1,3,2-oxathiaphospholane

Into the mixture of pyridine (79.1g, 1.0 mol) and benzene (400 mL) were added at room temperature, with stirring, 2-mercaptoethanol (39.1g, 0.5 mol) and phosphorus trichloride (68.7g, 0.5 mol). Strirring was continued for 0.5 h, pyridinium chloride was filtered off, and the filtrate was condensed under reduced pressure. Crude product was purified via distillation under reduced pressure, and the fraction boiling at 70-72°C/20 mmHg, was collected. ^{31}P NMR: δ205.0 ppm (benzene). Yield: 72%.

Example 2

N,N-diisopropylamino-1,3,2-oxathiaphospholane

Into the solution of the 2-chloro-1,3,2-oxathiaphospholane of Example 1 (28.5g, 0.2 mol) in n-pentane (300 mL) was added dropwise, at room temperature, with stirring, N,N-diisopropylamine (40.5g, 0.4 mol). After 0.5 h diisopropylamine hydrochloride was removed by filtration, solvent was evaporated under reduced pressure, and the product was distilled. The fraction collected at 70°C/0.1 mmHg was shown by means of ^{31}P NMR to be homogeneous. Yield: 70%. ^{31}P NMR: δ147.8 ppm (benzene). MS: m/z 207 (M^-, E.I., 15 eV).

Example 3

2-N,N-diisopropylamino-4,4-dimethyl-1,3,2-oxathiaphospholane

Into a solution of 20 mmoles of anhydrous pyridine in benzene (25 mL) was added at temperature of 0-5°C, dropwise, with stirring, a mixture of 10 mmoles of 2-methyl-2-mercaptopropanol-1 and 10 mmoles of phosphorus trichloride. The reaction mixture was then maintained at ambient temperature for 1 h. Pyridine hydrochloride was filtered off and the filtrate was concentrated under reduced pressure. Distillation gave 2-chloro-4,4-dimethyl-1,3,2-oxathiaphospholane, b.p. 48-52°C/0.1 mmHg. Ten mmoles of this compound were dissolved in 25 mL of n-hexane and to this solution 20 mmoles of N,N-diisopropylamine were added, with stirring, at room temperature. After 1 h diisopropylamine hydrochloride was removed by filtration and the product, 2-N,N-diisopropylamino-4,4-dimethyl-1,3,2-oxathiaphospholane, was distilled under reduced pressure. The fraction at 86-90°C/0.1 mmHg, ^{31}P NMR (C_6D_6) δ154.3 ppm, was collected.

Example 4

2-N,N-diisopropylamino-1,3,2-dithiaphosoholane

A. Into a solution of 20 mmoles of anhydrous pyridine in benzene (25 mL) was added at 0-5°C, dropwise, with stirring, simultaneously: 10 mmoles of 1,2-ethanedithiol and 10 mmoles of phosphorus trichloride. The reaction mixture was then stirred at room temperature for 1 h. Pyridine hydrochloride was filtered off and the filtrate was evaporated under reduced pressure. The residue was distilled at 14 mmHg to give 1.15 g (73%) 2-chloro-1,3,2-dithiaphospholane as a colorless liquid, b.p. 110°C. ^{31}P NMR: δ170.7 ppm (C_6D_6).

B. Into a solution of 2-chloro-1,3,2-dithiaphospholane (10 mmole) in benzene (25 mL) was added, dropwise at 0-5°C, with stirring, N,N-diisopropylamine (20 mmole). The reaction mixture was then stirred at room temperature for 1 h. Diisopropylamine hydrochloride was filtered off and the filtrate was evaporated under reduced pressure.

The residue was distilled at 0.6 mmHg yielding 1.4 g (63%) of 2-N,N-diisopropylamino-1,3.2-dithiaphospholane in the form of a colorless liquid, b.p. 110°C. ^{31}P NMR: δ93.9 ppm (CD_3CN)

Example 5

N^6-isopropoxyacetyl-5'-O-(4,4'-dimethoxytriphenylmethyl)-2 -deoxyadenosine-3'-O-[1,3,2-oxathiaphospholane-2-sulfide]

1 mmole N^6-isopropoxyacetyl-5'-O-DMT-2'-deoxyadenosine and 1 mmole 1-H-tetrazole were dried under vacuum at 50°C. After dissolving in anhydrous methylene chloride (3 mL) 2-diisopropylamino-1,3,2-oxathiaphospholane (1,1 mmole) was added with stirring. Stirring was continued for 1 h at ambient temperature and then 10 mmole of elemental sulfur was added and stirred overnight. The reaction mixture was concentrated to dryness and purified on a silica gel column. Yield: 90% ^{31}P NMR: δ103.63; δ103.58 ppm (C_6D_6), 85% H_3PO_4 as an external standard.

Example 6

N^4-isopropoxyacetyl-5'-O-DMT-2'-deoxycytidine-3'-O-[1,3,2-oxathiaphosoholane-2-sulfide]

The same procedure was followed as in Example 5, with the exception that N^4-isopropoxyacetyl-5'-O-DMT-2'-deoxycytidine -2'-deoxycytidine was used as starting material. Yield: 87-88%. ^{31}P NMR: δ104.38; δ104.36 ppm (C_6D_6), 85% H_3PO_4 as an external standard.

Example 7

N^2-isopropoxyacetyl-5'-O-DMT-2'-deoxyguanosine-3'-O-[1,3,2-oxathiaohosoholane-2-sulfide]

The same procedure was followed as in Example 5, with the exception that N^2-isopropoxyacetyl-5'-O-DMT-2'-deoxyguanosine was used as starting material. Yield: 72%. ^{31}P NMR: δ103.66; 6103.58 ppm (C_6D_6), 85% H_3PO_4 as an external standard.

Example 8

5'-O-DMT-2'-deoxythymidine-3'-O-[1,3,2-oxathiaphosphotane-2-sulfide]

The same procedure was followed as in Example 5, with the exception that 5'-O-DMT-2'-deoxythymidine was used as starting material. Yield: 92%. ^{31}P NMR: δ104.14; δ104.12 ppm (C_6D_6), 85% H_3PO_4 as an external standard.

Example 9

Separation of the Stereo-isomers of the Synthons of Examples 5-8

Diastereoisomeric mixtures of the synthons (A^{ipa}, C^{ipa}, G^{ipa}, and T represent the products of Examples 5-8, respectively) were separated on silica gel (Kieselgel 60H) columns (200 x 60 mm) as indicated in the table below. 1 g of the product was applied in each case.

			Faster Eluted Isomer		Slower Eiuted Isomer	
Synthon	Eluent	Ratio	Amt.	^{31}P NMR	Amt.	^{31}P NMR
A^{ipa}	ethyl acetate: heptane	2:1	300 mg	103.58	250 mg	103.68
C^{ipa}	ethyl acetate: methylene chloride	1:1	350 mg	104.36	200 mg	104.38
G^{ipa}	ethyl acetate: methylene chloride: methanol	2:1:0.2	230 mg	103.50	150 mg	103.66
T	ethyl acetate: heptane	2:1	350 mg	104.12	200 mg	104.14

Example 10

Further Synthesis of Nucleoside-3'-O-[2-thiono-1,3,2-oxathiaphospholane] Synthons

Nucleoside synthons were prepared with the exocyclic amines of the base moieties protected as follows: N^6-benzoyladenine, N^4-benzoylcytosine, and N^2-isobutyrylguanine (the corresponding synthons being represented as A^{bz}, C^{bz}, and G^{ibu}, respectively, and the thymidine synthon as T). Each of the four synthons were prepared as follows: The mixture of the respective protected 5'-DMT-O-(2'-deoxyribonucleoside) (10 mmol) and 1H-tetrazole (0.77g, 11 mmol) was dried under high vacuum for 5 h and then dissolved in dichloromethane (25 mL). Into this solution the product of Example 2 (2.28g, 11 mmol) was added dropwise over 10 min and the resulting mixture was maintained at room temperature with stirring for 2 h. Elemental sulfur (0.48g, 15 mmol), previously dried on a vacuum line for several hours, was added in one portion to the reaction mixture which was left overnight with stirring. Unreacted sulfur was filtered off and solvent was evaporated on a rotatory evaporator. The residue was dissolved in chloroform (3mL) and applied to a silica gel (30cm x 6cm column, 170g of 230-400 mesh silica). Elution was performed first with $CHCl_3$ (200 mL) and then with $CHCl_3$:CH_3OH (97:3, v/v). Isolation was monitored by HPTLC of collected fractions. Collected fractions containing the respective products were pooled tcgether and evaporated. After solvent evaporation, all products were obtained as white foamy solids and consisted of mixtures of the respective S_p and R_p diastereoisomers in yields of 90%, 89%, 85%, and 92% for A^{bz}, C^{bz}, G^{ibu}, and T, respectively).

The pure diastereoisomers in all cases were obtained by procedures similar to that which follows for C^{bz}. 1g of the C^{bz} product was dissolved in ethyl acetate (4 mL) and loaded onto a column (30cm x 6cm) of silicagel 60H (200g. Merck. Art. No. 7736). Diastereoisomers were eluted with ethyl acetate and fractions of 15 mL were collected. Elution of products was followed by means of HPTLC (threefold development in ethyl acetate; detection: HCl spray). Fractions containing separated diastereoisomers (FAST: fractions 61-73, and SLOW: fractions 87-98) were pooled together, respectively, concentrated to dryness under reduced pressure, and the residue was characterized by means of ^{31}P NMR and HPLC (using Lichrospher Si100,5 µm (30cm x 7.8mm), with ethyl acetate as an eluent-flow rate 3 mL/min). For the FAST diastereoisomer: 250 mg recovery (yield 25%). ^{31}P NMR (in C_6D_6, H_3PO_4 as an external standard): δ104.31 ppm (benzene) 100% diastereoisomeric purity. For the SLOW diastereoisomer: 180 mg recovery (yield 18%), ^{31}P NMR: δ104.26 ppm (benzene) 100% d.p. Fractions 74-86 were recycled for repeated isolations of the isomers. Data on the isolated synthons is summarized in the table below:

	Faster Eluted Isomer		Slower Eluted Isomer	
Synthon	^{31}P NMR	R_f	^{31}P NMR	R_f
A^{bz}	103.23	0.34	103.18	0.31
C^{bz}	104.31	0.27	104.26	0.22
G^{ibu}	104.52	0.22	104.17	0.20
T	104.27	0.59	104.23	0.57

Example 11

5'-O-DMT-thymidine-3'-O-[2-thiono-4,4-dimethyl-1,3,2-oxathiaphospholane

1 mmol of 5'-O-DMT-thymidine and 1 mmol of 1H-tetrazole were dried under vacuum for 3 h. 4 mL of CH_2Cl_2 were added and then the resulting solution was mixed with 1 mmol of 2-N,N-diisopropylamino-4,4-dimethyl-1,3,2-oxathiaphospholane. Phosphitylation was followed by means of TLC ($CHCl_3$:MeOH at 9:1). After disappearance of the phosphitylating reagent, to the reaction mixture 1 mmol of elemental sulfur was added and the reaction was stirred for 2 h at room temperature. Products were purified on a silicagel column using $CHCl_3$. Yield: 68%, R_f (TLC) 0.74 ($CHCl_3$: MeOH at 9:1)) ^{31}P NMR: 108.08; 107.80 ppm (CD_3CN).

Example 12

Nucleoside-5'-O-DMT-3'-O-[2-thiono-1,3,2-dithiaphospholane] Synthons

The product of Example 4 was reacted as described in Example 11 with 5'-DMT-protected nucleosides (exocyclic amines being protected as in Example 10) to give the 2-thiono-1,3,2-dithiaphospholane synthons. It was found that the effectiveness of the sulfurization was enhanced by the presence of trace amounts of pyridine. The results are given below:

Synthon	Yield	R_f (TLC)	^{31}P NMR ppm
A^{bz}	62.4%	0.80	123.95 (CD_3CN)
C^{bz}	81.4%	0.74	124.70 (CD_3CN)
G^{ibu}	50.4%	0.61	124.96 (CD_3CN)
T	74.4%	0.65	121.93 (C_6D_6)

Example 13

5'-O-DMT-thymidine-3'-O-[2-seleno-1,3,2-oxathiaphospholane]

5'-O-DMT-thymidine was reacted with 2-N,N-diisopropylamino-1,3,2-oxathiaphospholane and elemental selenium under conditions analogous to those of Example 11. Yield 80%, ^{31}P NMR: 99.05, 98.90 ppm, ($CHCl_3$), J_{PSe} 952.16 Hz, R_f 0.77 ($CHCl_3$:MeOH at 9:1).

Example 14

Synthesis of 5'-O-DMT-nucleosides Bound to Solid Phase Support Via Sarcosinyl Linker

A. Long chain alkylamine CPG (LCA-CPG, Sigma, Cat. No. L-8638, 500 A, 80-130 mesh, 2 g) and N-Fmoc-sarcosine (Bachem Bioscience, Inc., Prod. No. B-1720, 0.5 g, 1.6 mmol) were mixed together and dried under high vacuum for 3 h. Dry dimethylformamide (5 mL), pyridine (0.5 mL) and dicyclohexylcarbodiimide (0.5 g, 2.4 mmol) were added and the whole mixture placed in a tightly closes vial (7.4 mL) was gently shaken for 12 h. A suspension of the solid phase support was transferred into sintered glass funnel, solvent removed by suction and the support was washed three times with methanol/acetonitrile/pyridine (1:1:1, v/v/v, 3 x 20 mL). Residual solvents were removed under high vacuum and the N-Fmoc-sarcosinylated LCA-CPG was suspended in a 10% solution of piperidine in pyridine (v/v, 10 mL) for 0.5 h to remove the Fmoc protecting group. The N-sarcosinylated LCA-CPG was filtered off and washed with methanol/acetonitrile/pyridine (1:1:1, v/v/v, 3 x 20 mL) and subsequently dried under high vacuum for 5 h.

B. The product obtained according to A (0.5 g) was separately mixed with the respective 3'-O-succinylated 5'-O-DMT-dA^{bz}, -dG^{ibu}, -dC^{bz}, and -dT, and the mixtures were dried under high vacuum for 2 h, after which DMF (2 mL), pyridine (0.2 mL), and DCC (50 mg) were added and the resulting mixtures were moderately shaken at room temperature in tightly closed vials for 12 h. A suspension of the solid support from each mixture was separately transferred to a sintered glass funnel, washed three times with methanol/acetonitrile/pyridine (1:1:1, v/v/v, 3 x 20 mL), and finally with acetonitrile (3 x 10 mL). After drying with a flow of dry nitrogen, the supports were treated with an acylating reagent (N-methylimidazole/THF, 1 mL, Applied Biosystems, Inc. Cat. No. 400785, and acetic anhydride/lutidine/THF, 1 mL, Applied Biosystems, Inc. Cat. No. 400607) for 15 min. After a thorough wash with methanol/acetonitrile/pyridine (1:1:1, v/v/v, 3 x 10 mL) and acetonitrile (3 x 10 mL), the resulting solid phase supports were dried under high vacuum. Loading of the supports with the respective nucleoside as determined by trityl assay were as follows: CPG-sarcosinyl-dA^{bz}: 42.7 μmol/g; CPG-sarcosinyl-dG^{ibu}: 46.7 μmol/g; CPG-sarcosinyl-dC^{bz}: 31.6 μmol/g; and CPG-sarcosinyl-dT: 35.0 μmol/g.

Example 15

2'-deoxyadenylyl-(3',5')-2'-deoxyadenosine phosphorothioate (R_p isomer)

A. Through a column (volume 110 μL), secured with a filter at each end, and containing 1μmol of N_6-isopropoxyacetyl-5'-O-DMT-2'-deoxyadenosine (Uznanski et al, Nucleic Acids Research, Vol. 17, pgs. 4863-4868 (1989)) attached to a solid support, 5 mL of 2% dichloroacetic acid in methylene chloride was passed for 1 minute, then the support was washed with 15 mL of acetonitrile and dried under high vacuum.

B. Into the column prepared as described above, 110 μL of the solution containing 30 μmol of N_6-isopropoxyacetyl-5'-O-DMT-2'-deoxyadenosine-3'-O-1,3,2-oxathiaphospholane-2-sulfide (slower eluting diastereoisomer) and 300 μmol of 1,8-diazabicyclo[5.4.0]-undec-7-ene (DBU) was introduced. The reaction was continued for 20 minutes at 18-24°C. The column was washed with 10 mL of acetonitrile, and then 5 mL of 2% dichloroacetic acid in methylene chloride was passed in order to deprotect the 5'-hydroxyl function.

C. After a thorough wash with acetonitrile, 1 mL of 25% ammonia solution was slowly passed through the column

for 1 h in order to cleave the dimer from the solid support and to deprotect the exocyclic amino functions. The product was purified by means of HPLC (PRP1 Hamilton column, 30 x 7 mm) using a gradient of 5.20% acetonitrile-water, 0.1 M triethylammonium bicarbonate (TEAB), flow rate 3 mL/min.

Example 16

($R_pR_pR_pR_pR_pR_pR_pR_p$)-Octathymidyl phosphorothioic acid

The synthesis was performed as described in Example 14, except that 5'-O-DMT-thymidine-3'-O-1,3,2-oxathiaphospholane synthons (slower eluting diastereoisomers) were used in 7 coupling cycles. The product was washed, cleaved and deprotected, and purified as described in Example 14.

The product was assayed by snake venom phosphodiesterase (svPDE)(Crotalus adamanteus), which is known to hydrolyze R_p isomers of phosphorothioates, and by nuclease P1 (Penicilium citrinum), which is known to hydrolyze S_p isomers of phosphorothioates. Buffer I was prepared for the svPDE: 100 mM Tris HCl pH 8.5 and 15 mM $MgCl_2$. Buffer II was prepared for nuclease P1: 100 mM Tris HCl pH 7.2 1 mM $ZnCl_2$. For the svPDE assay, 1 optical density unit of the octathymidyl phosphorothioic acid product was added to 20 μg of svPDE in 500 μL of Buffer I and incubated for 24 h at 37°C. HPLC analysis as described in Example 14 showed that the product was completely hydrolyzed to thymidine-5'-phosphorothioate. For the nuclease P1 assay, 1 optical density unit of the octathymidyl phosphorothioic acid product was added to 10 μg of svPDE in 500 μL of Buffer II and incubated for 24 h at 37°C. HPLC analysis as described in Example 14 showed that the product was completely resistant to hydrolysis by the enzyme.

Example 17

($S_pS_pS_pS_pS_pS_pS_pS_p$)-Octathymidyl phosphorothioic acid

The completely S_p-chiral octathymidyl phosphorothioic acid was synthesized as in Example 15, except that the faster eluting (S_p) diastereoisomer of 5'-O-DMT-thymidine-3'-O-1,3,2-oxathiaphospholane-2-sulfide was used as the synthon. The product was assayed by svPDE and nuclease P1 as in Example 15 and was found to be completely resistant to svPDE and to be completely hydrolyzed by nuclease P1.

Example 18

Dithymidyl-(3',5')-phosphorodithioate

A. In solution.

The mixture of 5'-O-DMT-thymidine-3'-O-[2-thiono-1,3,2-dithiaphospholane] (0.3 mmol) and 3'-O-acetyl thymidine (0.3 mmol) was cried under vacuum for 3 h, then dissolved in 3 mL of anhydrous CH_3CN. To this solution 0.33 mmol of DBU was added and this mixture was maintained, with stirring, for 3 h at ambient temperature. ^{31}P NMR examination showed presence of 70% of 3'-O-5'-O-protected dithymidyl-(3',5')-phosphorodithioate (δ116.7 ppm), 2% of unreacted synthon, and 28% of side products (δ71.8 and δ72.2 ppm). Evaporation of solvent left solid which was dissolved in 3 mL of 80% CH_3COOH. After 2 h acetic acid was removed under reduced pressure and residue was redissolved in 5 mL of 25% NH_4OH. This solution was incubated at 55°C for 15 h. After concentration and dissolution in 5 mL of water, solid particles were filtered off and filtrate was introduced into a column filled with DEAE-Sephadex A-25. Product was eluted with TEAB, gradient 0.05-1 M. UV-absorbing fraction was collected and concentrated. ^{31}P NMR (D_2O) showed the presence of product, δ113.8 ppm, purity >95%, Yield from UV absorption profile, 46.6%. Further analysis by FAB-MS confirmed product as dithymidyl-(3',5')-phosphorodithioate.

B. On solid support.

1 μmol of CPG-T in a column (Applied Biosystems, Inc.) was detritylated and 10 μmol of 5'-O-DMT-thymidine-3'-O-[2-thiono-1,3,2-dithiaphospholane] (dried under vacuum) diluted with 140 μL of CH_3CN was introduced to the column together with 10 μmol of DBU in 15 μL of CH_3CN. After 10 minutes (with occasional shaking), the column was washed with CH_3CN, detritylated, and deprotected and cleaved using standard procedures. HPLC analysis (ODS Hypersil, linear gradient 5->20% CH_3CN in 0.1 M TEAB, 20 min, R.T. 12.3 min.) showed product consisted of dithymidyl phosphorodithioic acid contaminated with 8% thymidine.

Example 19

Dithymidyl-(3',5')-phosphoroselenoate

A. In solution.

0.1 mmol of the product from Example 13 in 0.5 mL CH_3CN was added to the mixture of 0.1 mmol 3'-O-methoxyacetyl thymidine and 0.15 mmol DBU. After 10 min ^{31}P NMR examination showed the presence of about 80% of 3',5'-protected dithymidyl-(3',5')-phosphoroselenoate (δ49.7 and δ49.5 ppm) and 20% of unidentified side products (δ59.7 and δ59.6 ppm).

B. On a solid support.

1 μmol of CPG-T in a column (Applied Biosystems, Inc.) was detritylated and 10 μmol of 5'-O-DMT-thymidine-3'-O-[2-seleno-1,3,2-oxathiaphospholane] diluted with 50 μL CH_3CN was introduced to the column together with 20 μmol of DBU in 100 μL of pyridine. After 10 min, the column was washed with acetonitrile, detritylated, and the product was cleaved from the support. HPLC analysis (ODS Hypersil, linear gradient 5->20% CH_3CN in 0.1 M TEAB over 20 min. R.T. of 8.87 and 9.54. Yield: 85% (via HPLC).

Example 20

Control of Stereospecificity under Conditions of Automated Solid Phase Synthesis

An Applied Biosystems, Inc. (Foster City, CA, USA) model 380B automated DNA synthesizer was employed using the manufacturer's columns (1 μmol scale). The manufacturer's program used routinely for the synthesis of oligonucleotides via the 2-cyanoethylphosphoramidite method was modified according to the protocol presented in the following table:

Chemical steps for one synthesis cycle				
Step	Reagent or Solvent		Purpose	Time (min)
1	a) Dichloroacetic acid in CH_2Cl_2 (2:98, v/v)	2 mL	DETRITYLATION	1.5
	b) Acetonitrile	5 mL	WASH	2
2	a) Activated nucleotide in acetonitrile*		COUPUNG	1 0
	b) Acetonitrile	5 mL	WASH	2
3	a) Acetic anhydride/lutidine in THF (10: 10:80, v/v/v)	1 mL	CAPPING	1
	N-methylimidazole in THF (16:84, v/v)	1 mL		
	b) Acetonitrile	5 mL	WASH	1

* For 1 μmol synthesis scale 2 M DBU in pyridine (150 μL), and 0.1 M 5'-O-DMT-deoxynucleoside-3'-O-[2-thiono-1,3,2-oxathiaphospholane] (of Example 10)(50 μL) in acetonitrile was used.

Reservoirs 1-4 of the DNA synthesizer were filled with solutions of pure diastereoisomers (Example 10) in acetonitrile (0.1 M), and reservoir 9 (usually containing the 1H-tetrazole activator) was filled with 2 M DBU in pyridine. In each case, at the completion of synthesis, acidic detritylation was followed by ammonia cleavage and base deprotection. Products (dimers) were analyzed and purified by reverse phase HPLC using an ODS Hypersil (5 μm) column (30 cm x 4.6 mm) that was eluted with the linear gradient of acetonitrile: 5->20% CH_3CN/0.1 mol TEAB; 0.75%/min; flow rate 1.5 mL/min. Results are summarized in the table below, HPLC profiles are given in Stec et al, Nucleic Acids Research, Vol. 19, pgs. 5883-5888 (1991):

Stereospecificities of the formation of dinucleotide-(3',5')-phosphorothioates			
Synthon (a)	Diastereomeric purity (b)	Product	Diastereomeric purity (c)
T	FAST 100%	$[S_p]$ -d(TT)	99.0%
T	SLOW 100%	$[R_p]$-d(TT)	100.0%
A^{bz}	FAST 100%	$[S_p]$-d(AA)	99.4%

(continued)

Stereospecificities of the formation of dinucleotide-(3',5')-phosphorothioates			
Synthon (a)	Diastereomeric purity (b)	Product	Diastereomeric purity (c)
A^{bz}	SLOW 100%	$[R_p]$-d(AA)	99.5%
C^{bz}	FAST 1 00%	$[S_p]$-d(CC)	99.3%
C^{bz}	FAST 95% (d)	$[S_p]$-d(CC)	95.0%
C^{bz}	SLOW 100%	$[R_p]$-d(CC)	99.5%
C^{bz}	SLOW 95% (d)	$[R_p]$-d(CC)	95.0%
G^{ibu}	FAST 100%	$[S_p]$-d(GG)	99.3%
G^{ibu}	SLOW 100%	$[R_p]$-d(GG)	98.0%
(a) Same nomenclature as in Example 10.			
(b) All the diastereoisomeric synthons were identified via HPLC by coinjections with genuine samples prepared according to Stec et al, J. Amer. Chem. Soc., Vol. 106, pgs. 6077-6079 (1984).			
(c) Via HPLC.			
(d) Prepared by mixing separated diastereoisomers.			

As can be seen from the above data, the SLOW eluting diastereoisomers are always giving a dinucleotide product in the R_p configuration, while the FAST eluting diastereoisomers are always giving a dinucleotide product in the S_p configuration.

Example 21

Control of Stereospecificity under Conditions of Automated Solid Phase Synthesis Using Nucleosides Bound to Solid Phase Support Via Sarcosinyl Linker

The procedures of Example 20 are followed, except that in place of the manufacturer's columns, columns are provided that are filled with 5'-DMT-nucleosides bound to a solid phase support via a sarcosinyl linker as described in Example 14.

Example 22

($R_pR_pR_pR_pR_p$)-penta-(2'-O-deoxycytidine phosphorothioate)

A ($R_pR_pR_pR_pR_p$)-penta-(2'-O-deoxycytidine phosphorothioate) was synthesized with an automated DNA synthesizer as described in Example 19 using the CPG-sarcosinyl-dC^{bz} solid phase support as described in Example 20. A solution of the SLOW eluting synthon of Example 10 (100 mg) in acetonitrile (1.2 mL) was employed. Tritylated and detritylated products were isolated by two-step reverse phase HPLC. Analysis of both the tritylated and detritylated products by HPLC (same column as in Example 19) gave rise to single peaks (flow rate 1.5 mL/min). For tritylated compound: r.t. 20.40 min (5-30% CH_3CN/0.1 M TEAB, t=20 min, exponent 0.25). For detritylated product: r.t. 11.80, 5-20% CH_3CN/0.1 M TEAB, 0.75%/min). Preparative yield: 14%. Enzymatic analysis as described above showed the product to be of all-R_p form.

Example 23

($S_pS_pS_pS_pS_p$)-penta-(2'-O-deoxycytidine phosphorothioate)

A ($S_pS_pS_pS_pS_p$)-penta-(2'-O-deoxycytidine phosphorothioate) was prepared as described in Example 21, except that the FAST eluting synthon was employed. With the same analysis as in Example 21, the tritylated compound had a r. t. of 20.7 min, and the detritylated product had a r.t. of 12.00 min. Preparative yield: 15%. Enzymatic analysis as described above showed.the product to be of all-S_p form.

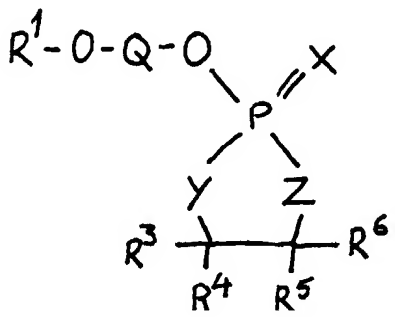
Example 24

Synthesis of P-chiral Antisense Comoound

All-R_p and all-S_p forms of the 28-mer antisense oligonucleotide phosphorothioate, 5' 5'-TCGTCGCTGTCTCCGCT-TCTTCCTGCCA, are synthesized with an automated DNA synthesizer as described by Examples 19 and 20 using the SLOW and FAST eluting synthons of Example 10, respectively.

Claims

1. A compound of the general formula



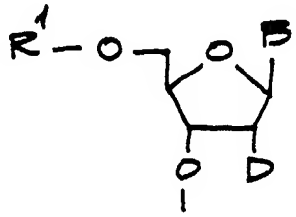
in which

Q is C_1-C_8 alkylene, C_2-C_8 alkenylene or C_1-C_8 oxaalkylene or thia-alkylene each containing 1 or 2 heteroatoms, or -O-Q-O- is a nucleoside or nucleoside analog;
R^1 is hydrogen or a hydroxyl protecting group;
X is O, S, Se or $=NR^2$ in which R^2 is C_1-C_6 alkyl or C_6-C_{12} aryl, alkylaryl or alkenylaryl;
Y is O, S or Se;
Z is S or Se; and

each of R^3, R^4, R^5 and R^6 is hydrogen or C_1-C_4 alkyl or all of R^3, R^4, R^5 and R^6 and the carbon atoms to which they are attached together constitute C_6-C_{12} aryl, alkylaryl or alkenylaryl.

2. A compound according to claim 1, in which Q is C_3-C_6 alkylene, alkenylene, or oxa-alkylene.

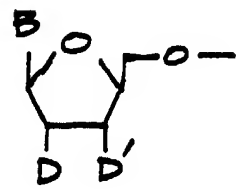
3. A compound according to claim 1 or claim 2, in which R^1-O-Q-O- is a group of the formula



in which

B is a purine or pyrimidine base;
D is hydrogen, halogen, hydroxyl or OR' in which R' is C_1-C_3 alkyl or a 2'-hydroxyl protecting group; and
R^1 has the meaning specified in claim 1.

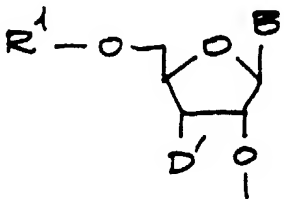
4. A compound according to claim 1 or claim 2, in which R^1-O-Q-O- is a group of the formula



in which

D' is hydrogen, hydroxy or OR" in which R" is a 3'-hydroxyl protecting group; and
B and D have the meanings specified in claim 3.

5. A compound according to claim 1 or claim 2, in which R^1-O-Q-O- is a group of the formula



in which R^1 has the meaning specified in claim 1, B has the meaning specified in claim 3 and D' has the meaning specified in claim 4.

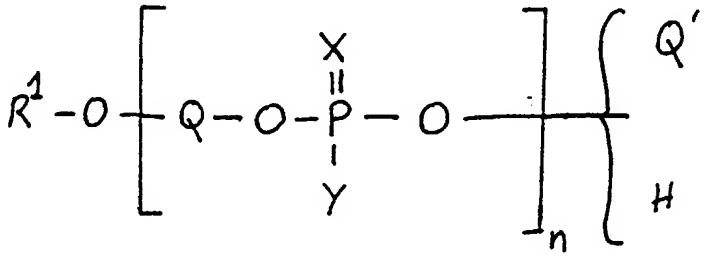
6. A compound according to any one of claims 1 to 5, which is in the diastereoisomerically pure R_p form.

7. A compound according to any one of claims 1 to 5, which is in the diastereoisomerically pure S_p form.

8. A method for the preparation of a compound according to any one of claims 1 to 7 in the presence of a reagent [x], wherein

(i) X is O and [X] is elemental oxygen or a peroxide;
(ii) X is S and [X] is elemental sulfur, an acyl disulfide or a diphosphorothioyl disulfide;
(iii) X is Se and [X] is elemental selenium or potassium selenocyanate; or
(iv) X is=NR^2 and [X] is N_3R^2 in which R^2 has the meaning specified in claim 1.

9. A polymeric compound comprising a sequence of monomer units and of phosphorus linkages of specified chirality, the polymeric compound having the general formula

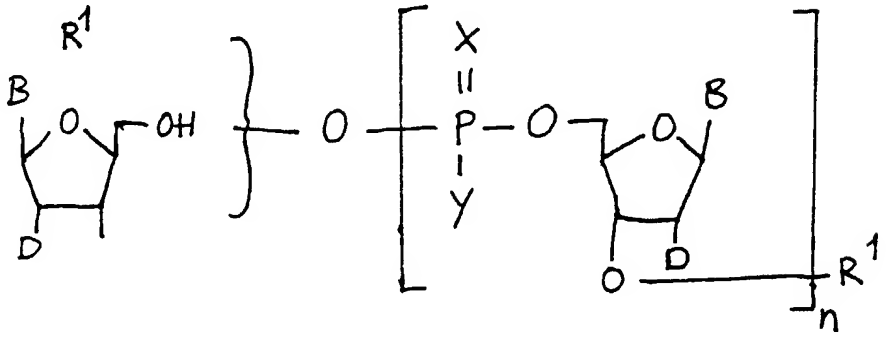


in which

Q' is C_1-C_8 alkyl, C_2-C_8 alkenyl or C_1-C_8 oxa-alkyl or thia-alkyl each containing 1 or 2 heter-

oatoms, or -O-Q' is a nucleoside or nucleoside analog;
n has a value from 5 to 200; and
Q, R^1, X and Y have the meanings specified in claim 1.

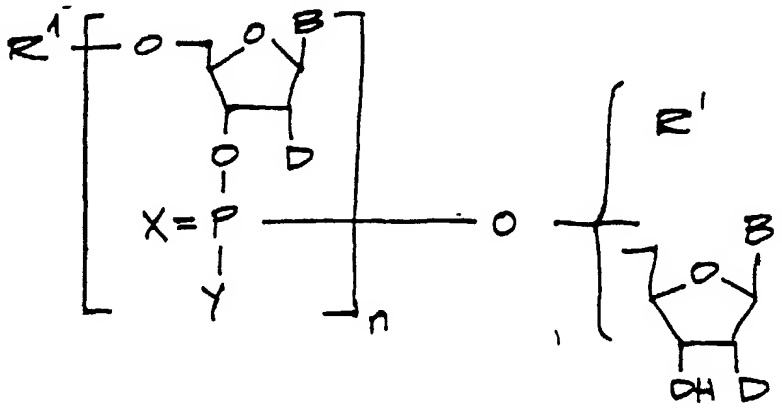
10. A polymeric compound according to claim 9, having the general formula



in which

R^1, X and Y have the meanings specified in claim 1; and
B and D have the meanings specified in claim 3.

11. A polymeric compound according to claim 9, having the general formula



in which

R^1, X, and Y have the meanings specified in claim 1; and
B and D have the meanings specified in claim 3.

12. A polymeric compound according to claim 11, wherein

X is O or S; and
Y is O or S.

13. A polymeric compound according to claim 11 or claim 12, wherein n has a value from 12 to 60.

14. A polymeric compound according to any one of claims 11 to 13, wherein all phosphorus linkages are R_p chiral.

15. A polymeric compound according to any one of claims 11 to 13, wherein all phosphorus linkages are S_p chiral.

16. A polymeric compound according to claim 14 or claim 15, in the form of a 21-mer to 28-mer oligonucleotide having the sequence

5'-TCGTCGCTGTCTCCGCTTCTTCCTGCCA.

17. A method for the preparation of a polymeric compound comprising a sequence of monomer units and of phosphorus linkages and having the general formula specified in claim 9, which comprises the steps of

(a) attaching a first monomer having a protected hydroxyl to a solid phase support;
(b) deprotecting the protected hydroxyl to form a free hydroxyl;
(c) reacting the free hydroxyl with a compound according to any one of claims 1 to 7; and
(d) repeating steps (b) and (c) until a product of desired length is obtained.

18. A method according to claim 17 for the preparation of a polymeric compound according to any one of claims 9 to 16, wherein in step (c) the compound according to any one of claims 1 to 7 is of specified chirality.

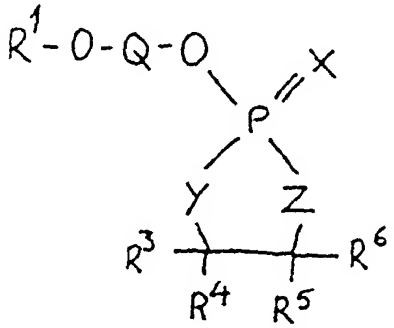
19. A method according to claim 17 or claim 18, which includes the further step of capping unreacted free hydroxyls after step (c).

20. A method according to any one of claims 17 to 19, wherein step (c) is carried out in the presence of a catalytic base.

21. A method according to claim 20, wherein the catalytic base is 1,8-diazabicyclo[5.4.0]undec-7-ene.

Patentansprüche

1. Verbindung der allgemeinen Formel



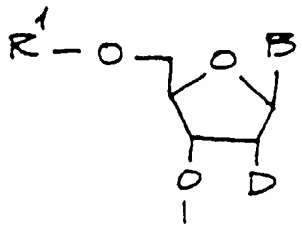
worin

- Q für C_1-C_8-Alkylen, C_2-C_8-Alkenylen oder C_1-C_8-Oxaalkylen oder -Thiaalkylen steht, die jeweils 1 oder 2 Heteroatome enthalten, oder -O-Q-O- ein Nukleosid oder Nukleosidanalogon bedeutet,
- R^1 Wasserstoff oder eine Hydroxy-Schutzgruppe bedeutet,
- X für O, S, Se oder $=NR^2$ steht, worin R^2 C_1-C_6-Alkyl oder C_6-C_{12}-Aryl, Alkylaryl oder Alkenylaryl bedeutet,
- Y für O, S oder Se steht,
- Z für S oder Se steht und

jedes von R^3, R^4, R^5 und R^6 Wasserstoff oder C_1-C_4-Alkyl bedeutet oder alle Gruppen R^3, R^4, R^5 und R^6 und die Kohlenstoffatome, an die sie gebunden sind, zusammen C_6-C_{12}-Aryl, -Alkylaryl oder -Alkenylaryl darstellen.

2. Verbindung nach Anspruch 1, worin Q C_3-C_6-Alkylen, -Alkenylen oder -Oxaalkylen bedeutet.

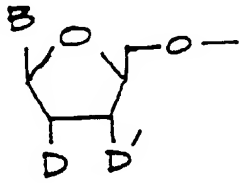
3. Verbindung nach Anspruch 1 oder Anspruch 2, in der R^1-O-Q-O- eine Gruppe der Formel



darstellt, worin

B eine Purin- oder Pyrimidinbase bedeutet,
D Wasserstoff, Halogen, Hydroxyl oder OR' ist, wobei R' C_1-C_3-Alkyl oder eine Schutzgruppe für die 2'-Hydroxygruppe darstellt, und
R^1 die in Anspruch 1 angegebene Bedeutung hat.

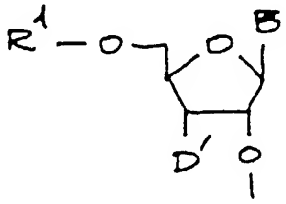
4. Verbindung nach Anspruch 1 oder Anspruch 2, worin R^1-O-Q-O- eine Gruppe der Formel



darstellt, worin

D' Wasserstoff, Hydroxyl oder OR" bedeutet, wobei R" eine Schutzgruppe für die 3'-Hydroxygruppe darstellt und
B und D die in Anspruch 3 angegebene Bedeutung haben.

5. Verbindung nach Anspruch 1 oder Anspruch 2, worin R^1-O-Q-O- eine Gruppe der Formel



bedeutet, worin R^1 die in Anspruch 1 angegebene Bedeutung hat, B die in Anspruch 3 angegebene Bedeutung hat und D' die in Anspruch 4 angegebene Bedeutung hat.

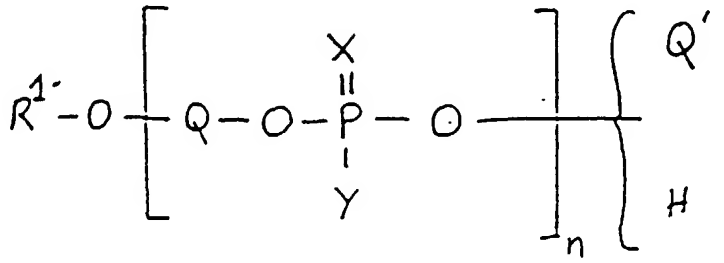
6. Verbindung nach einem der Ansprüche 1 bis 5, die in der Form des reinen R_P-Diastereoisomeren vorliegt.

7. Verbindung nach einem der Ansprüche 1 bis 5, die in der Form des reinen S_p-Diastereoisomeren vorliegt.

8. Verfahren zur Herstellung einer Verbindung nach einem der Ansprüche 1 bis 7 in Gegenwart eines Reagenzes [X], worin

(i) X für O steht und [X] elementarer Sauerstoff oder ein Peroxid ist,
(ii) X für S steht und [X] elementarer Schwefel, ein Acyldisulfid oder ein Diphosphorothioyldisulfid ist,
(iii) X für Se steht und [X] elementares Selen oder Kaliumselenocyanat ist oder
(iv) X für $=NR^2$ steht und [X] N_3R^2 ist, worin R^2 die in Anspruch 1 angegebene Bedeutung hat.

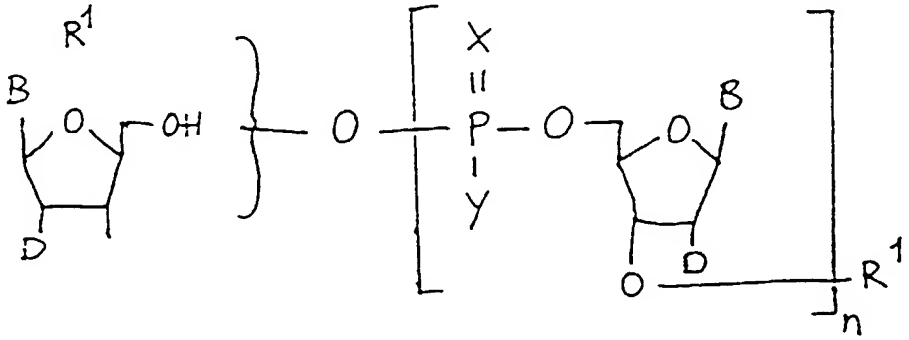
9. Polymere Verbindung, die eine Sequenz von Monomereinheiten und von Phosphorverknüpfungen mit spezifischer Chiralität aufweist, wobei die polymere Verbindung die allgemeine Formel



hat, in der

Q'	für C_1-C_8-Alkyl, C_2-C_8-Alkenyl oder C_1-C_8-Oxaalkyl oder -Thiaalkyl steht, die jeweils 1 oder 2 Heteroatome enthalten, oder -O-Q' ein Nukleosid oder Nukleosidanalogon bedeutet,
n	einen Wert von 5 bis 200 hat und
Q, R^1, X und Y	die in Anspruch 1 angegebene Bedeutung haben.

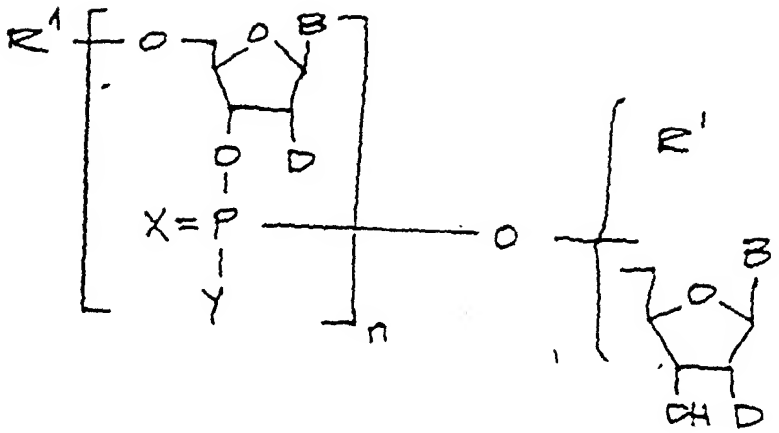
10. Polymere Verbindung nach Anspruch 9 der allgemeinen Formel



worin

R^1, X und Y	die in Anspruch 1 angegebene Bedeutung haben und
B und D	die in Anspruch 3 angegebene Bedeutung haben.

11. Polymere Verbindung nach Anspruch 9 der allgemeinen Formel



worin

R^1, X und Y die in Anspruch 1 angegebene Bedeutung haben und
B und D die in Anspruch 3 angegebene Bedeutung haben.

12. Polymere Verbindung nach Anspruch 11, worin

X für O oder S steht und
Y für O oder S steht.

13. Polymere Verbindung nach Anspruch 11 oder Anspruch 12, worin n einen Wert von 12 bis 60 hat.

14. Polymere Verbindung nach einem der Ansprüche 11 bis 13, wobei alle Phosphorverknüpfungen R_p-chiral sind.

15. Polymere Verbindung nach einem der Ansprüche 11 bis 13, worin alle Phosphorverknüpfungen S_p-chiral sind.

16. Polymere Verbindung nach Anspruch 14 oder Anspruch 15 in Form eines 21-meren bis 28-meren Oligonukleotids der Sequenz

5′-TCGTCGCTGTCTCCGCTTCTTCCTGCCA.

17. Verfahren zur Herstellung einer polymeren Verbindung, die eine Sequenz von Monomereinheiten und von Phosphorverknüpfungen umfaßt und die in Anspruch 9 angegebene allgemeine Formel aufweist, welches folgende Stufen umfaßt:

(a) Binden eines ersten Monomeren, das eine geschützte Hydroxylgruppe aufweist, an einen Festphasenträger,
(b) Entfernen der Schutzgruppe von der geschützten Hydroxylgruppe unter Bildung einer freien Hydroxylgruppe,
(c) Umsetzen der freien Hydroxylgruppe mit einer Verbindung nach einem der Ansprüche 1 bis 7 und
(d) Wiederholen der Stufen (b) und (c) bis ein Produkt der gewünschten Länger erhalten worden ist.

18. Verfahren nach Anspruch 17 zur Herstellung einer polymeren Verbindung nach einem der Ansprüche 9 bis 16, wobei die Verbindung nach einem der Ansprüche 1 bis 7 in Stufe (c) eine spezifische Chiralität aufweist.

19. Verfahren nach Anspruch 17 oder Anspruch 18, welches die weitere Stufe des Verkappens von nicht umgesetzten freien Hydroxylgruppen nach Stufe (c) umfaßt.

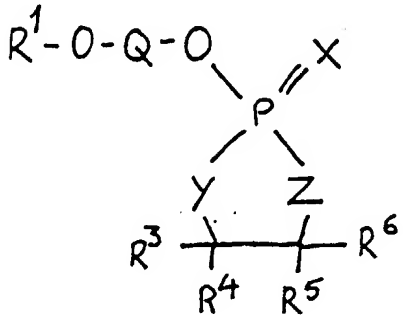
20. Verfahren nach einem der Ansprüche 17 bis 19, wobei Stufe (c) in Gegenwart einer katalytischen Base durchge-

führt wird.

21. Verfahren nach Anspruch 20, wobei die katalytische Base 1,8-Diazabicyclo[5.4.0]undec-7-en ist.

Revendications

1. Composé représenté par la formule générale :

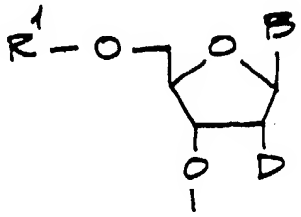


dans laquelle :

- Q représente alkylène en C_1-C_8, alcénylène en C_2-C_8 ou oxa-alkylène ou thia-alkylène en C_1-C_8, contenant chacun 1 ou 2 hétéroatomes, ou -O-Q-O- est un nucléoside ou un analogue de nucléoside ;
- R^1 représente hydrogène ou un groupe protecteur d'hydroxyle ;
- X représente O, S, Se ou $=NR^2$, où R^2 représente alkyle en C_1-C_6 ou aryle, alkylaryle ou alcénylaryle, en C_6-C_{12} ;
- Y représente O, S ou Se ;
- Z représente S ou Se ; et
- R^3, R^4, R^5 et R^6 représentent chacun hydrogène ou alkyle en C_1-C_4 ou bien la totalité des radicaux R^3, R^4, R^5 et R^6 et les atomes de carbone auxquels ils sont attachés constituent ensemble aryle, alkylaryle ou alcénylaryle, en C_6-C_{12}.

2. Composé selon la revendication 1, dans lequel Q représente alkylène, alcénylène ou oxa-alkyléne en C_3-C_6.

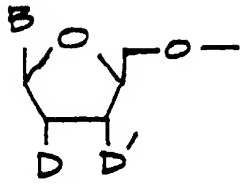
3. Composé selon la revendication 1 ou la revendication 2, dans lequel R^1-O-Q-O- est un groupe de la formule :



dans laquelle :

- B représente une base purique ou pyrimidinique ;
- D représente hydrogène, halogène, hydroxyle ou OR', où R' représente alkyle en C_1-C_3 ou un groupe protecteur de 2'-hydroxyle ; et
- R^1 a la signification spécifiée dans la revendication 1.

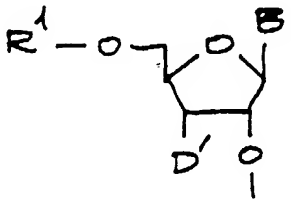
4. Composé selon la revendication 1 ou la revendication 2, dans lequel R^1-O-Q-O- est un groupe de la formule :



dans laquelle :

- D' représente hydrogène, hydroxy ou OR", où R" est un groupe protecteur de 3'-hydroxyle ; et
- B et D ont les significations spécifiées dans la revendication 3.

5. Composé selon la revendication 1 ou la revendication 2, dans lequel R^1-O-Q-O- est un groupe de la formule :



dans laquelle :

- R^1 a la signification spécifiée dans la revendication 1 ;
- B a la signification spécifiée dans la revendication 3 ; et
- D' a la signification spécifiée dans la revendication 4.

6. Composé selon l'une des revendications 1 à 5, qui se trouve dans la forme R_p diastéréoisomériquement pure.

7. Composé selon l'une des revendications 1 à 5, qui se trouve dans la forme S_p diastéréoisomériquement pure.

8. Procédé pour la préparation d'un composé tel que défini à l'une des revendications 1 à 7, en présence d'un réactif [X], dans lequel :

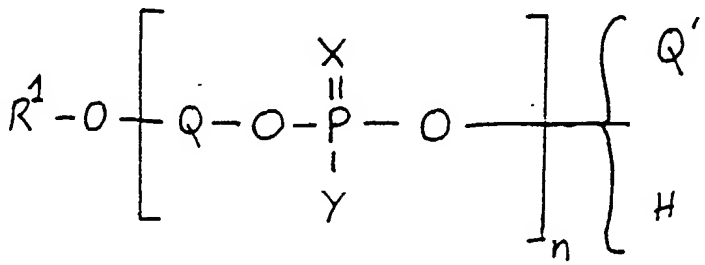
(i) X représente O et [X] est l'oxygène élémentaire ou un peroxyde ;

(ii) X représente S et [X] est le soufre élémentaire, un disulfure d'acyle ou un disulfure de diphosphorothioyle ;

(iii) X représente Se et [X] est le sélénium élémentaire ou le sélénocyanate de potassium ; ou

(iv) X représente $=NR^2$ et [X] représente N_3R^2 où R^2 a la signification spécifiée dans la revendication 1.

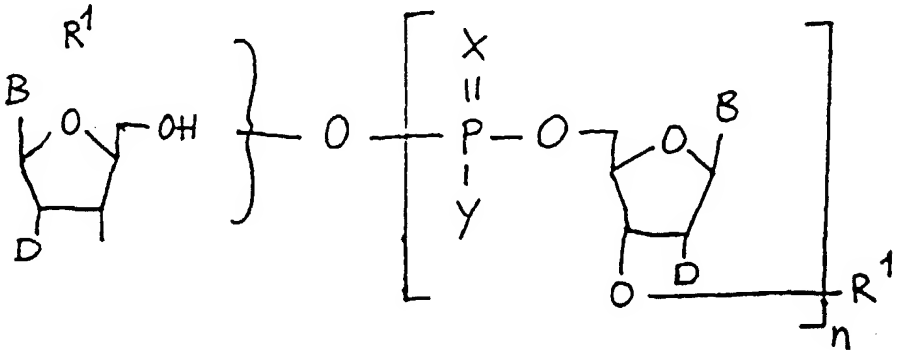
9. Composé polymère comprenant une séquence d'unités monomères et de liaisons phosphore de chiralité spécifiée, le composé polymère ayant la formule générale :



dans laquelle :

- Q' représente alkyle en C_1-C_8, alcényle en C_2-C_8, ou oxa-alkyle ou thia-alkyle en C_1-C_8, contenant chacun 1 ou 2 hétéroatomes, ou -O-Q' représente un nucléoside ou un analogue de nucléoside ;
- n a une valeur allant de 5 à 200 ; et
- Q, R^1, X et Y ont les significations spécifiées dans la revendication 1.

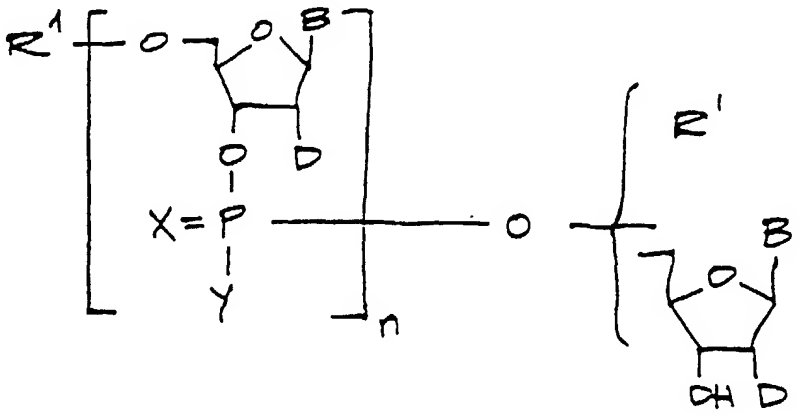
10. Composé polymère selon la revendication 9, ayant la formule générale :



dans laquelle :

- R^1, X et Y ont les significations spécifiées dans la revendication 1 ; et
- B et D ont les significations spécifiées dans la revendication 3.

11. Composé polymère selon la revendication 9, ayant la formule générale :



dans laquelle :

- R^1, X et Y ont les significations spécifiées dans la revendication 1 ; et
- B et D ont les significations spécifiées dans la revendication 3.

12. Composé polymère selon la revendication 11, dans lequel :

- X représente O ou S ; et
- Y représente O ou S.

13. Composé polymère selon la revendication 11 ou la revendication 12, dans lequel n a une valeur de 12 à 60.

14. Composé polymère selon l'une des revendications 11 à 13, dans lequel toutes les liaisons phosphore sont de chiralité R_p.

15. Composé polymère selon l'une des revendications 11 à 13, dans lequel toutes les liaisons phosphore sont de chiralité S_p.

16. Composé polymère selon la revendication 14 ou la revendication 15, se présentant sous la forme d'un oligonucléotide 21-mère à 28-mère ayant la séquence :

5'-TCGTCGCTGTCTCCGCTTCTTCCTGCCA .

17. Procédé de préparation d'un composé polymère comprenant une séquence d'unités monomères et de liaisons phosphore et ayant la formule générale spécifiée dans la revendication 9, qui comprend les étapes consistant à :

(a) fixer un premier monomère ayant un hydroxyle protégé sur un support en phase solide ;

(b) déprotéger l'hydroxyle protégé afin de former un hydroxyle libre ;

(c) faire réagir l'hydroxyle libre avec un composé tel que défini à l'une des revendications 1 à 7 ; et

(d) répéter les étapes (b) et (c) jusqu'à ce qu'un produit de longueur désirée soit obtenu.

18. Procédé selon la revendication 17 pour la préparation d'un composé polymère tel que défini à l'une des revendications 9 à 16, dans lequel, dans l'étape (c), le composé tel que défini à l'une des revendications 1 à 7 est de chiralité spécifiée.

19. Procédé selon la revendication 17 ou la revendication 18, qui comprend l'étape supplémentaire de coiffage des hydroxyles libres n'ayant pas réagi après l'étape (c).

20. Procédé selon l'une des revendications 17 à 19, dans lequel l'étape (c) est effectuée en présence d'une base catalytique.

21. Procédé selon la revendication 20, dans lequel la base catalytique est le 1,8-diazabicyclo[5.4.0]undec-7-ène.